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Title:

Characterization of MtnE, the fifth metallothionein member in *Drosophila*

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Abstract

Metallothioneins (MTs) constitute a family of cysteine-rich, low molecular weight metal-binding proteins which occur in almost all forms of life. They bind physiological metals, such as zinc and copper, as well as non-essential, toxic heavy metals, such as cadmium, mercury and silver. MT expression is regulated at the transcriptional level by the metal-responsive transcription factor-1 (MTF-1), which binds to the metal response elements (MREs) in the enhancer/promoter regions of MT genes. *Drosophila* was thought to have four MTs, namely *MtnA*, *MtnB*, *MtnC* and *MtnD*. Here we characterize a new fifth member of *Drosophila* MT gene family, coding for metallothionein E (*MtnE*). The *MtnE* transcription unit is located head-to-head with the one of *MtnD*. The intervening sequence contains four MREs which bind, with different affinities, to MTF-1. Both the divergently transcribed metallothionein genes are completely dependent on MTF-1, whereby *MtnE* is consistently more strongly transcribed. *MtnE* expression is induced in response to heavy metals, notably copper, mercury and silver, and is upregulated in a genetic background where the other four metallothioneins are missing.

Keywords: metallothionein E, *Drosophila*, MTF-1, metal response element, cadmium toxicity

Introduction

Metallothioneins (MTs) are cysteine-rich, low molecular weight proteins that are able to bind a wide range of metals including cadmium, zinc, mercury, copper and silver [1-5]. MTs were first described in 1957 by Vallee and Margoshes as cadmium-binding proteins in the horse renal cortex [6]. MTs play an essential role in heavy metal detoxification and in maintaining the homeostasis of essential trace metals. They are also involved in the protection against free radicals and oxidative stress [7-9]. MTs are ubiquitously found in eukaryotes and also in some prokaryotes [10]. Humans have at least one dozen MT genes, while the mouse has four. *Drosophila* was thought to have four MTs (*MtnA-D*) expressed mainly in the digestive tract [11]. *S. cerevisiae* has two MTs, *CUP1* and *CRS5* [12, 13].

MTF-1 is a zinc-finger containing transcription factor conserved from insects to humans and is the main regulator of the expression of metallothioneins [reviewed in 14-16]. MTF-1 plays a role not only in heavy metal stress but also in other cell stress conditions, such as oxidative stress and hypoxia [17-20]. MTF-1 was discovered in 1988 and shown to be a protein which requires elevated zinc concentrations for optimal DNA binding [21]. Subsequently the mouse gene was cloned and characterized as a ubiquitously expressed zinc finger transcription factor essential for basal and heavy metal-induced expression of metallothioneins [22, 23]. The cloning of *MTF-1* genes of human, fish and *Drosophila* followed [24-27]. *MTF-1* null mutant mouse embryos develop severe liver degeneration and die *in utero* at approximately day 14 of gestation [28]. Conditional *MTF-1* knockout mice in which the gene is

disrupted in the liver and hematopoietic tissues are viable, but highly sensitive to cadmium exposure and display reduced leukocyte counts [29]. In contrast to the lethal phenotype of the mouse *MTF-1* null mutant, *MTF-1* knockout *Drosophila* are viable and fertile under standard laboratory conditions, but sensitive to heavy metal load and to copper starvation [30]. *Drosophila* MTF-1 is essential for the basal and metal-induced expression of the metallothionein genes [30]. Moreover, it is also active under copper starvation where it mediates expression of the intestinal copper importer *Ctr1B* [31]. Transcriptome analysis also revealed *ferritins*, the ABC transporter *CG10505*, the zinc transporter *ZnT35C* and glutathione *S*-transferase genes (*GstD2* and *GstD5*) as target genes of *Drosophila* MTF-1 [32]. The involvement of the latter two genes is in agreement with findings in mammals, in that both MTF-1/metallothioneins and glutathione play a role in counteracting metal stress [33].

Mammalian MTF-1 has a molecular mass of approximately 75 kDa (mouse and human MTF-1 are 675 and 753 amino acids long, respectively). The N-terminal domain is followed by six Cys²-His² type zinc fingers. Mammalian MTF-1, besides nuclear localization and nuclear export signals, contains three activation domains: an acidic one, a proline-rich one and a serine/threonine-rich one [34]. A conspicuous cysteine cluster (CQCQCAC) near the C-terminus is also required for transcriptional activity [35] and mediates homodimerization of human MTF-1 [V. G. and W. S., unpublished data]. Recently it was shown that the nuclear localization signal (NLS) of human MTF-1 is a non-conventional one, spanning zinc fingers 1-3 within the DNA-binding domain [36]. *Drosophila* MTF-1 cDNA encodes a protein of 791 amino acids; it shares 39% amino acid similarity with human MTF-1 and has a particularly striking similarity in the region of all six zinc fingers to its vertebrate homologs [27].

However, whereas human *MTF-1* shows a strong induction in response to zinc and cadmium, *Drosophila MTF-1* is best activated by copper and cadmium [27].

As already mentioned, MTF-1 regulates the expression of metallothioneins at the transcriptional level [21-23]. Upon metal stress, MTF-1 binds to short DNA sequences in the enhancer/promoter regions of metallothionein genes and other target genes, termed metal-response elements (MREs). MREs consist of a highly conserved core consensus sequence, (t)TGCRCNC (R = A or G, and N = any nucleotide), followed by less conserved GC-rich sequences [37-41]. A synthetic promoter composed of several MRE sequences is sufficient for MTF-1 to drive expression of a gene upon metal induction [42]. Mutations of single nucleotides in MRE sequences can reduce or abolish its function [22].

Here we describe the fifth *Drosophila* metallothionein gene, annotated as *CG42872* in Flybase (www.flybase.org). It encodes a protein that is highly similar to the other *Drosophila* metallothioneins, especially MtnB, C and D [11, 30], and was named metallothionein E (MtnE) (Figure 1a and b). *CG42872 (MtnE)* was discovered by targeted cDNA screening of gene predictions as part of the modENCODE transcriptome project [43]. The captured cDNA MIP01063 provided evidence for 5' and 3' UTRs and allowed characterization of the upstream regulatory region of the gene (Figure 2a). Similar to other metallothionein genes, *MtnE* possesses upstream MRE sequence motifs which are apparently shared with *MtnD* (Figure 2b and c). We cloned the genomic region containing the *MtnE* and *MtnD* genes, which are adjacent to each other in a head-to-head orientation. In order to study the expression profiles,

MtnD and *MtnE* were differentially tagged with GFP and/or mCherry fluorescent tags. *MtnE* is induced by several heavy metals and its expression, like that of the divergently transcribed *MtnD*, is completely dependent on MTF-1.

Figure 1 a, b,

Figure 2 a, b, c

Materials and Methods

Annotation of MT genes in insects

MT-type genes in the different insect genomes were identified by standard BLAST searches (NCBI BLAST and Flybase BLAST) against the sequenced genomes and against protein databases. Sequence alignments were generated with the CLC Sequence Viewer, Version 6.3, with standard parameters.

Fly culture

One liter of fly food was composed of 55 g cornmeal (Maisgriess 54.401.025, Meyerhans Hotz AG), 100 g yeast (Hefe Schweiz AG), 75 g sugar (Dextrose monohydrate), 8 g agar (Insectagar type ZN5) and 15 ml anti-fungal Nipagin (nipagin 33 g/l, nipasol 66 g/l in 96% ethanol). For experiments, food was supplemented with

CuSO₄, HgCl₂, AgNO₃ or ZnSO₄ to the concentrations indicated on the figures. Flies were raised at 25°C in 65% humidity.

Tissue preparation and microscopy

For the whole-larvae imaging, wandering 3rd instar larvae were placed on glass slides and immobilized by incubating them shortly (~1 min) at 4°C. The pictures were taken using a Leica DMRB microscope and a Zeiss Axiocam color camera with 2.5x magnification.

To harvest guts, wandering 3rd instar larvae were dissected in ice-cold PBS, pH 7.4. Tissues were directly mounted in *Drosophila* Ringer's solution (3 mM CaCl₂, 46 mM NaCl, 182 mM KCl, 10 mM Tris, pH 7.2) and microphotographed. FITC and TRITC filter epifluorescence pictures from larval guts were taken using a Zeiss Axioplan 2 microscope and a Zeiss Axiocam MRm camera with 5x magnification.

Plasmid construction and transgenic fly strains

Transgenic fly lines were generated using ϕ C31-mediated transgenesis [44, 45]. In all MT expression experiments the *86Fb* (chromosome 3R) landing site was used for plasmid integration. Flies carrying the transgene integration site also expressed the ϕ C31 integrase, which was removed by crossing out after the integration event occurred. To generate *MtnD* and *MtnE* transgenes, a DNA segment containing the two transcription units and flanking sequences corresponding to the segment of *D.*

melanogaster of coordinates 3R: complement (16360941..16363020) was cloned into a vector containing an *attB* site for integration into the specific genomic locus, and a *miniwhite* marker gene. For the overexpression of *Drosophila* MTF-1, we used the constitutively active *Drosophila* tubulin promoter (*Tub*). The VSV-tagged transgene (*Tub-dMTF-1-VSV*) was integrated into the *51D* locus on the 2nd chromosome. The null allele for *dMTF-1* is termed *D4*.

For bacterial expression, first the coding region of *MtnE* was obtained using coupled RT-PCR technology (QIAGEN). Then, the PCR product was introduced to pET-24a (Novagen), where *MtnE* is expressed under the T7 bacteriophage promoter. Plasmid sequences and detailed cloning strategies are available upon request.

Cadmium toxicity assay in *E.coli*

E. coli BL21 (DE3) were transformed with the *MtnE*-expression or pET-24a control vector. Liquid cultures (LB-medium + kanamycin) were grown to an OD₆₀₀ between 0.4 - 0.5 and expression of *MtnE* was induced by 0.4 mM IPTG for two hours. Cadmium was added to the indicated concentrations and bacteria were grown at 37 °C. After 21 hours the OD₆₀₀ was determined.

Electrophoretic mobility shift assay

Drosophila Schneider S2 cells were transiently transfected with the respective constructs and collected 48 hours later. Transfected cells were incubated in medium

containing 400 μM CuSO_4 prior to harvesting. Electrophoretic mobility shift assays (EMSAs) were performed as described by Radtke *et al* [22] and Zhang *et al* [27]. Binding reactions were performed by incubating 20 fmoles of [γ - ^{32}P]ATP end-labeled, 24-bp-long double stranded DNA oligonucleotides containing the MRE sequences, with nuclear extracts prepared according to Schreiber *et al* [46]. The following MRE oligonucleotides (core consensus sequences TCGRCNC indicated in capital letters) were used for EMSA:

MRE 1: 5' - gattttttgGTGCGCAaagcagt - 3'
 3' - aaaaaacCACGCGTtctgtcactt - 5'

MRE 2: 5' - taaagcgattTGCACACgccctga - 3'
 3' - tcgctaaACGTGTGcgggactatt - 5'

MRE 3: 5' - gtcgatcggcGTGCGCAaaagcat - 3'
 3' - ctagccgCACGCGTtttcgtaaaa - 5'

MRE 4: 5' - taagaaagccGTGTGCAagtcgat - 3'
 3' - ctttcggCACACGTtcagctagcc - 5'

MRE-s: 5' - cgaggagctcTGCACACggcccgaaaagtg - 3'
 3' - tcgagctccctcgagACGTGTGccgggcttttcacagct - 5'

Results

***MtnE* transcription is induced by several heavy metals and dependent on MTF-1**

Transgenic larvae carrying a genomic construct encoding a GFP- or mCherry-tagged *MtnE* gene showed stronger fluorescence in the presence of heavy metals, namely,

copper, mercury and silver (Figure 3). An elevated expression of *MtnE* was also observed when the larvae were raised on food supplemented with 1 mM zinc while iron had at most a marginal effect on *MtnE* expression (data not shown).

Figure 3

The expression of *MtnE*'s partner gene *MtnD* was also metal responsive, whereby the *MtnE* fusion transgene yielded an even stronger fluorescence than *MtnD* both on NF and Cu-containing food (Figure 4).

Figure 4

Overexpression of *MTF-1* (in a heterozygous *MTF-1* mutant background) greatly enhanced the expression of *MtnE* (Figure 5a). In an homozygous *MTF-1* mutant background, basal and copper induced expression of *MtnE* is undetectable (Figure 5b). From these results we conclude that the expression of *MtnE*, like the other members of *Drosophila* MT family, is regulated by MTF-1. Knockout of the other four members of the MT family enhances basal as well as induced expression of *MtnE*, indicating a compensatory upregulation (Figure 5b).

Figure 5 a, b

Expression patterns of *MtnE* and *MtnD* genes largely overlap

We further investigated the expression of MtnE-GFP and MtnD-mCherry proteins in *Drosophila* larval tissues. Like that of other *Drosophila* metallothioneins, *MtnE* expression was observed in the intestine of larvae on normal food (Figure 6a) and was boosted by copper-containing food (Figure 3, Figure 6b). The expression patterns of *MtnD* and *MtnE* mostly overlap in the *Drosophila* larval gut, but there are some

differences: *MtnE* is more widely expressed than *MtnD* and *MtnB*, the latter of which is strongly expressed in a sub-region of the middle midgut that contains “copper cells” [11]. Copper cells are known to accumulate metal ions following copper or cadmium intoxication [11, 47, 48]. As indicated by blue arrows, copper also boosts the expression of *MtnD* in copper cells, while *MtnE* remains less induced. *MtnD* is also constantly expressed in the so-called iron cell region [49], independent of heavy metal load (Figure 6a, 6b).

According to RNA-seq data obtained with standard food [43], basal expression of *MtnE* essentially follows the pattern of *MtnB*, *C* and *D*, while expression of *MtnA* in the absence of metals is always higher (Table 1).

Figure 6

Table 1

Metal response elements shared by of *MtnD* and *MtnE* bind MTF-1 *in vitro*

As mentioned before, there are four MRE sequence motifs in the DNA segment between the divergently oriented *MtnD* and *MtnE* transcription units that are most likely shared functionally by these two genes. We studied the MTF-1 binding properties of single MREs in EMSA to elucidate their potential for metal-induced expression of *MtnD* and *MtnE*. MRE2, 3 and 4 clearly bind MTF-1, though not as strongly as the synthetic consensus sequence (MRE-s) (Figure 7).

Figure 7

Evidence for enhanced cadmium resistance of *MtnE*-expressing bacteria

To test whether MtnE could act as a metal scavenger in a heterologous system, we produced recombinant *E. coli* expressing *MtnE* from an IPTG-inducible T7 bacteriophage promoter. All clones were adjusted to the same OD, the lac-promoter driving MtnE was induced and bacteria were grown in liquid culture of standard medium containing different concentrations of CdCl₂. The next day, OD₆₀₀ was determined for each sample. Indeed upon *MtnE* induction bacteria displayed a relatively higher cadmium resistance than controls (Figure 8).

Figure 8

Discussion

The family of *Drosophila* metallothioneins is completed with a new member, *MtnE*. All five MT genes in *Drosophila* are located on the same chromosome and they harbour many structural and regulatory similarities. Here we show that *MtnE*, like the four previously described MTs, is expressed primarily in the intestine and in response to copper and cadmium. The *MtnE* gene shares a cluster of metal response elements with the divergently transcribed *MtnD*, and similar to the other MTs, its expression depends on MTF-1. It was shown before that flies with the quadruple knockout of *MtnA-D* genes are viable and fertile but sensitive to elevated concentrations of copper or cadmium, to a lesser extent to zinc, but not to mercury or silver load or copper depletion. The new member *MtnE* might complement the function of the others by widening the spectrum of metals that can be detoxified. Flies without *MTF-1* are much more sensitive to copper load than flies that lack four of its target genes, metallothioneins A-D. Our present results help to explain this observation, especially

since *MtnE* basal and copper-induced expression levels are elevated in the quadruple metallothionein background. This enhanced expression of the last remaining metallothionein gene is most likely a compensatory upregulation.

So far we have tested the resistance to cadmium of recombinant bacteria expressing *Drosophila MtnE* and found them to be more resistant than control bacteria.

Interestingly, *MtnE* expressors apparently grew even better in cadmium-loaded medium than in standard medium. The phenomenon where an organism thrives better under a (usually mild) stress condition than in a stress-free standard condition is known as hormesis. The putative hormesis effect observed for *MtnE*-producing *E. coli* is likely due to the metabolic cost of producing a protein with no function unless the organism is exposed to metal stress, whereupon the protein's presence becomes advantageous.

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Table Legends

Table 1.

Expression profiles of metallothionein genes according to RNA-seq data.

Figure Legends

Figure 1. *D. melanogaster* metallothioneins (*MtnA-E*)

- a) Sequence alignment of protein-coding cDNA regions of metallothioneins, which are all located on *D. melanogaster* chromosome 3R.
- b) Protein sequence alignment of MtnA-E. Note that MtnB, C, D and E sequences are most closely related to each other.

Figure 2. Metal response elements (MREs) of *MtnA-E* and genomic organization of *MtnD* and *MtnE*

- a) Scheme of *MtnD* and *MtnE* genomic region, including mRNA untranslated regions (grey) and protein coding sequences (colored). The *CG42872 (MtnE)* model is based on the cDNA MIP01063. There are alternative 3' UTRs for *MtnD* and *MtnE*.
- b) Sequence of the *MtnD* and *MtnE* genomic region, including transcription units and the regulatory regions. Four MREs are located between the transcription start sites (TSS). MREs are indicated in red and named MRE 1-4; nucleotides in capital letters are sequences found in cDNA isolates, blue letters indicate the protein-coding region.
- c) Schematic view of all five *Drosophila* metallothionein genes located on the R arm of chromosome 3. For each gene the transcription direction is indicated (black arrow). Core MRE nucleotides are depicted in red, with an open arrow indicating the MRE's orientation. Note that the scale bar of 20 nt is valid only for solid lines between the MREs, but not for the lettered DNA sequences themselves.

Figure 3. *MtnE-GFP* and *MtnE-mCherry* transgenes are expressed mostly in the larval digestive tract

Transgenic larvae were raised on normal food (NF), or food containing copper (Cu), mercury (Hg) or silver (Ag). Pictures with three representative larvae each were taken with 175 ms and 100 ms exposure time for mCherry- and GFP-tagged MTs, respectively. The concentration of metal ions in fly food is indicated on the top of each panel.

Figure 4. *MtnE* is more strongly transcribed than *MtnD*

In each picture, the larva on the left has the *MtnD* transgene tagged with mCherry or GFP, while the larva on the right carries *MtnE-mCherry* or *MtnE-GFP* transgenes. A longer exposure time (350 ms) was chosen for the upper panel, i.e., for larvae grown on NF, compared to larvae grown on Cu-food (150 ms), to demonstrate better the relative difference between *MtnD* and *MtnE* expression levels.

Figure 5. Expression of *MtnE* is dependent of MTF-1

- a) Expression of the *MtnE-GFP* transgene is stronger when MTF-1 is overexpressed (on an *MTF-1* heterozygous background; *D4* denotes the disrupted *MTF-1* locus) in comparison to the control without an *MTF-1* transgene.
- b) Expression of *MtnE* is completely dependent on MTF-1. *y w*, *MTF-1* *-/-* or *qMtn* (quadruple knockout of *MtnA-D*) larvae were raised on NF or Cu-food and transcript levels of endogenous *MtnE* was measured by S1-nuclease protection assay. Signals were normalized to *Actin 5c* levels and error bars indicate standard deviations of three experiments.

Figure 6. Expression pattern of metallothionein reporter constructs

Larvae were raised on normal food (a) or food containing 100 μ M copper (b). Fluorescence intensities are only comparable within parts (a) or (b) and there only within the same (red or green) fluorescent tag. Copper (cuprophilic) cells are indicated with blue arrows; orange arrows indicate the region of iron cells/midgut constriction. Three representative gut dissections are shown for each condition.

Figure 7. MTF-1 binds to the metal response elements of *MtnD/MtnE* genes

Drosophila S2 cells were transfected with mouse MTF-1 expression plasmids, and 10 μ g of nuclear protein extract was used for each bandshift reaction. Lanes 1-4, bandshift (EMSA) with [γ - 32 P]-labeled oligonucleotides representing MRE 1-4, respectively; lane 5, bandshift with a strong consensus binding site called MRE-s. For oligonucleotide sequences see Materials and Methods.

Figure 8. Survival of recombinant bacteria expressing *Drosophila MtnE*

Separate bacterial colonies were tested for growth in cadmium-containing LB-medium. Control 1 and 2: recombinant clones carrying “empty” plasmid vector (pET-24a); early stop 1 and 2: clones with a premature stop codon after the 12th amino acid, still including 4 out of 10 cysteines and thus expected to produce a mild protective effect; MtnE clones 1-4: full-length *MtnE* constructs. Bacteria were grown to the same OD₆₀₀ between 0.4 - 0.5, for induction IPTG was added. Two hours later cadmium was added at the indicated concentration and bacteria were grown for 21 hours.

Tables

Table 1.

Gene	0-2 hr embryos	14-16 hr embryos	L1 larvae	pupae	adult male	adult female
MtnA	4.3	318.9	1939.1	166.2	746.1	151.6
MtnB	0.1	0.0	235.3	133.7	2.1	17.9
MtnC	0.0	0.1	13.2	12.9	20.8	10.0
MtnD	0.1	0.2	94.1	15.5	4.7	10.6
MtnE	0.1	0.7	239.8	9.0	4.4	6.0

Figures

Figure 1.

a)

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MtnA CDS  ATG C C T T G C C  CA - - - T G C G G  A A G C G G A T G C  A A A T G C G C C A  G C C A G G C C A C  C A A G G G A T C C  57
MtnB CDS  ATG G T T T G C A  A G G G T T G T G G  A A C A A A C T G C  C A G T G C T C G G  C C C A A A A G T G  C G G G G A C A A C  60
MtnC CDS  ATG G T T T G C A  A A G G C T G C G G  A A C A A A C T G C  A A G T G C C A G G  A C A C C A A G T G  C G G C G A C A A T  60
MtnD CDS  ATG G G T T G C A  A G G C T T G T G G  A A C A A A C T G C  C A G T G C T C C G  C C A C C A A G T G  C G G T G A C A A C  60
MtnE CDS  ATG C C T T G C A  A G G G A T G T G G  A A A C A A C T G C  C A G T G C T C A G  C C G G A A A G T G  C G G A G G T A A C  60

MtnA CDS  T G C A A C T G C G  G A T C T G A C T G  C A A G T G C G G C  G G C G A C A A G A  A A T C C G C C  - - - - - T G C G G C  111
MtnB CDS  T G C G C C T G C A  A C A A G G A T T G  C C A G T G C G T T  T G C A A G A A T G  G G C C C A A G G A  C C A G T G C T G C  120
MtnC CDS  T G C G C C T G T A  A T C A G G A C T G  C A A G T G C G T G  T G C A A G A A T G  G C C C C A A A G A  T C A G T G T T G C  120
MtnD CDS  T G C G C C T G C A  G C C A G C A G T G  C C A G T G C T C C  T G C T A G A A C G  G A C C C A A G G A  C A A G T G C T G C  120
MtnE CDS  T G C G C C G G A A  A C A G C C A A T G  C C A A T G C G C C  G C C A A G A C G G  G A G C C A A G  - - - - - T G C T G C  114

MtnA CDS  T G C T C C G A G - - - T G A  123
MtnB CDS  A G C A A C A A A - - - T A A  132
MtnC CDS  A A G A G C A A G - - - T A G  132
MtnD CDS  T C C A C C A A A A  A C T A G  135
MtnE CDS  C A G G C C A A G - - - T G A  126

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b)

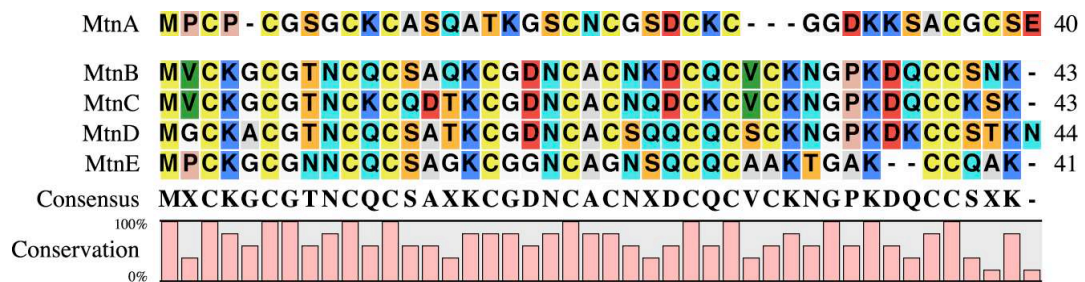
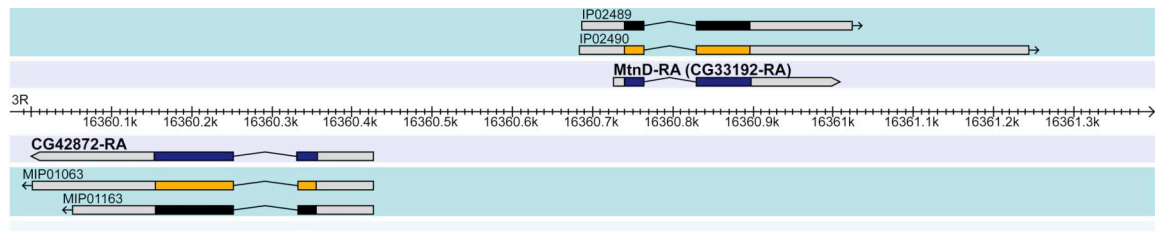


Figure 2.

a)



b)

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c)

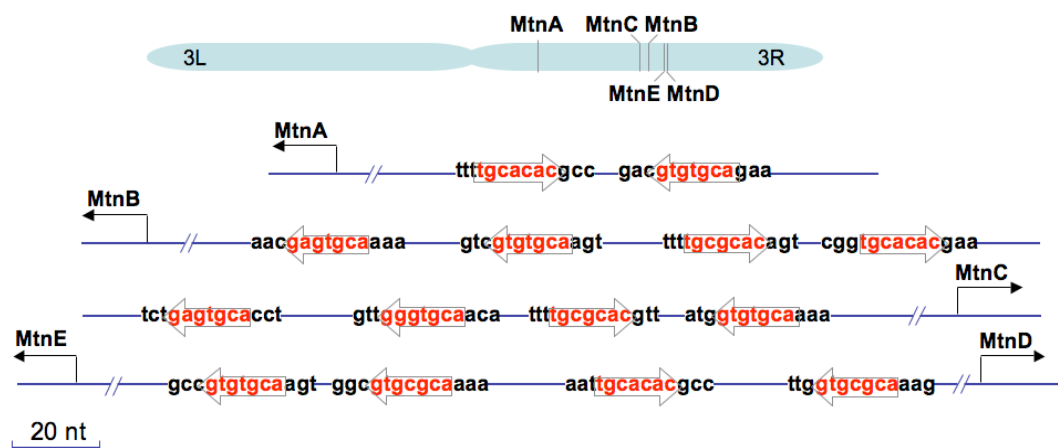


Figure 3.

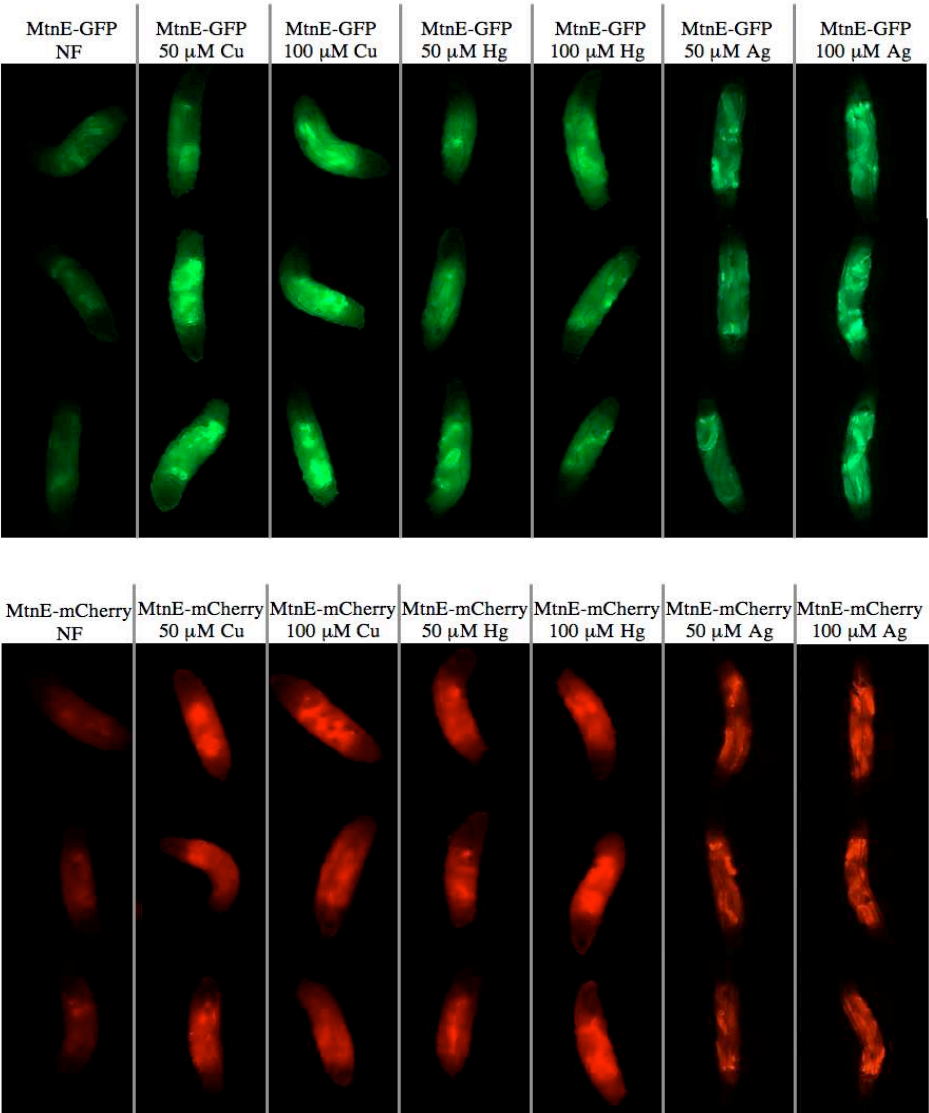


Figure 4.

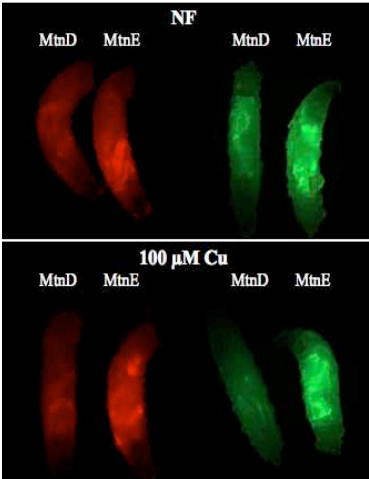
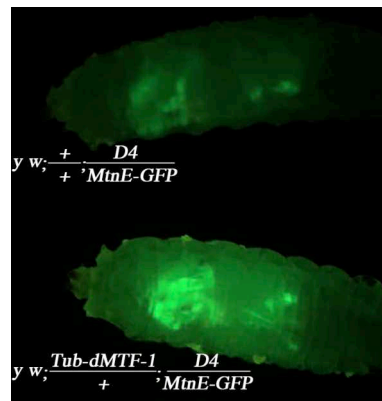


Figure 5.

a)



b)

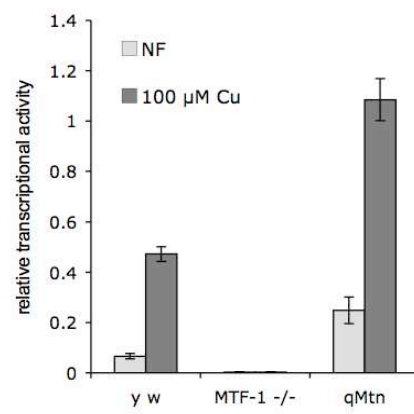
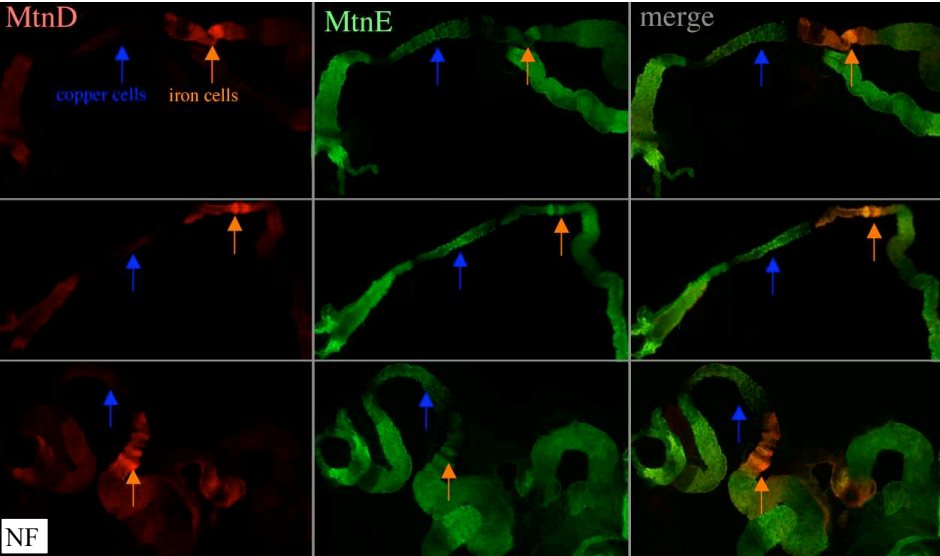


Figure 6.

a)



b)

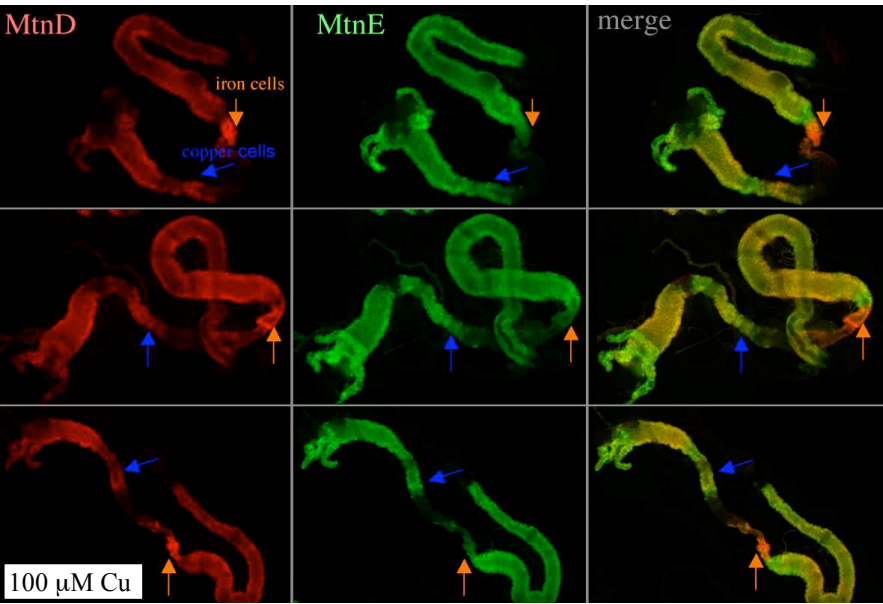


Figure 7.

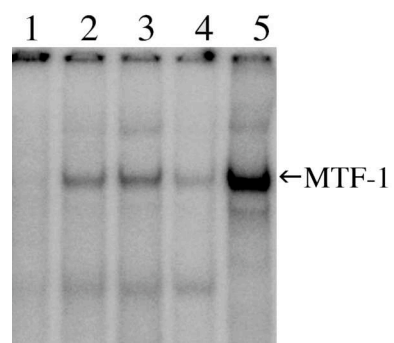


Figure 8.

